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Transformation-Mediated Complementation of a *FUM* Gene Cluster Deletion in *Fusarium verticillioides* Restores both Fumonisin Production and Pathogenicity on Maize Seedlings

Anthony E. Glenn,[1] Nicholas C. Zitomer,[1] Anne Marie Zimeri,[1] Lonnie D. Williams,[1,2] Ronald T. Riley,[1,2] and Robert H. Proctor[3]

[1]United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Russell Research Center, Toxicology & Mycotoxin Research Unit, Athens, GA 30605, U.S.A.; [2]Department of Environmental Health Science, University of Georgia, Athens 30602, U.S.A.; [3]USDA-ARS, National Center for Agricultural Utilization Research (NCAUR), Mycotoxin Research Unit, Peoria, IL 61604, U.S.A.

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The filamentous ascomycete *Fusarium verticillioides* is a pathogen of maize and produces the fumonisin mycotoxins. However, a distinct population of *F. verticillioides* is pathogenic on banana and does not produce fumonisins. Fumonisin-producing strains from maize cause leaf lesions, developmental abnormalities, stunting, and sometimes death of maize seedlings, whereas fumonisin-nonproducing banana strains do not. A Southern analysis of banana strains did not detect genes in the fumonisin biosynthetic gene (*FUM*) cluster but did detect genes flanking the cluster. Nucleotide sequence analysis of the genomic region carrying the flanking genes revealed that the *FUM* cluster was absent in banana strains except for portions of *FUM21* and *FUM19*, which are the terminal genes at each end of the cluster. Polymerase chain reaction analysis confirmed the absence of the cluster in all banana strains examined. Cotransformation of a banana strain with two overlapping cosmids, which together contain the entire *FUM* cluster, yielded fumonisin-producing transformants that were pathogenic on maize seedlings. Conversely, maize strains that possess the *FUM* cluster but do not produce fumonisins because of mutations in *FUM1*, a polyketide synthase gene, were not pathogenic on maize seedlings. Together, the data indicate that fumonisin production may have been lost by deletion of the *FUM* cluster in the banana population of *F. verticillioides* but that fumonisin production could be restored by molecular genetic complementation. The results also indicate that fumonisin production by *F. verticillioides* is required for development of foliar disease symptoms on maize seedlings.

Additional keywords: Gibberella fujikuroi species complex, *Gibberella moniliformis, Zea mays*

Corresponding author: A. E. Glenn; Telephone: +1.706.546.3119; Fax: +1.706.546.3116; E-mail: anthony.glenn@ars.usda.gov

Present address of A. M. Zimeri: Department of Environmental Health Science, University of Georgia, Athens, GA 30602, U.S.A.

Present address of L. D. Williams: Burdock Group, 2001 9th Ave, Suite 301, Vero Beach, FL 32960, U.S.A.

Nucleotide sequence data for the aberrant *FUM* locus of strain NRRL 25059 is available in the NCBI GenBank database under accession number EF653133.

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The filamentous ascomycete *Fusarium verticillioides* (teleomorph, *Gibberella moniliformis*) is consistently associated with maize (*Zea mays*) worldwide and is a food safety concern due to its production of various mycotoxins. Contamination of field-grown maize with the mycotoxin fumonisin B_1 (FB1) is of greatest concern because of its causal role in equine leukoencephalomalacia (Marasas et al. 1988), porcine pulmonary edema (Colvin and Harrison 1992), liver and renal carcinogenicity in laboratory rodents (IARC 2002), and possible human carcinogenicity (IARC 2002) and neural tube birth defects (Marasas et al. 2004). Fumonisins B_2, B_3, and B_4 (FB2, FB3, and FB4) also occur in maize but usually at concentrations lower than FB1.

Fumonisins are similar in structure to the free sphingoid bases sphinganine and sphingosine that are found in sphingolipids. The structural similarity between free sphinganine and fumonisins led to the discovery that fumonisins are potent and specific inhibitors of the acyl CoA-dependent ceramide synthase (Wang et al. 1991), a key enzyme in de novo sphingolipid biosynthesis. Sphingolipids are important for maintaining membrane structure and are involved in signal transduction pathways, cell cycle progression, and cell-to-cell recognition (Cheng et al. 2001; Merrill et al. 2001; Riley et al. 2001). Animal diseases caused by fumonisins are a consequence of disrupted sphingolipid metabolism (Marasas et al. 2004; Merrill, et al. 2001; Riley et al. 2001). Toxicity of fumonisins to plant cells also is associated with inhibition of acyl CoA-dependent ceramide synthase. FB1 and the structurally similar AAL toxin inhibit acyl CoA-dependent ceramide synthase in tomato and maize plants (Abbas et al. 1994; Riley et al. 1996; Williams et

al. 2006). AAL toxin is produced by *Alternaria alternata* f. sp. *lycopersici* (Gilchrist et al. 1992) and is essential for the fungus to cause stem cankers on susceptible tomato plants (Abbas et al. 1994; Brandwagt et al. 2000; Spassieva et al. 2002). Several major diseases of maize, including seedling blight, stalk rot, and ear rot, are attributed to *F. verticillioides* (Kommedahl and Windels 1981; White 1999), and the potential role of fumonisin production on development of these maize diseases has been investigated with varying results (Abbas and Boyette 1992; Desjardins and Plattner 2000; Desjardins et al. 1995, 1998, 2002; Doehlert et al. 1994; Lamprecht et al. 1994; van Asch et al. 1992; Williams et al. 2006, 2007). In relation to maize seedling blight, Desjardins and associates (1995) suggested that fumonisins increased the virulence of *F. verticillioides* but were not necessary or sufficient for disease development. Yet, Williams and associates (2006, 2007) reported a significant positive correlation between leaf lesion development on maize seedlings and the production of FB1 by *F. verticillioides*. They also showed a significant inverse correlation between root weight and stalk height and the amount of FB1 associated with seedling roots. Fumonisin-nonproducing strains did not cause leaf lesions and had significantly less effect on root weight and stalk height. Watering uninoculated maize seedlings with FB1 also caused a significant dose-dependent reduction in root and stalk development and induced leaf lesions (Williams et al. 2007).

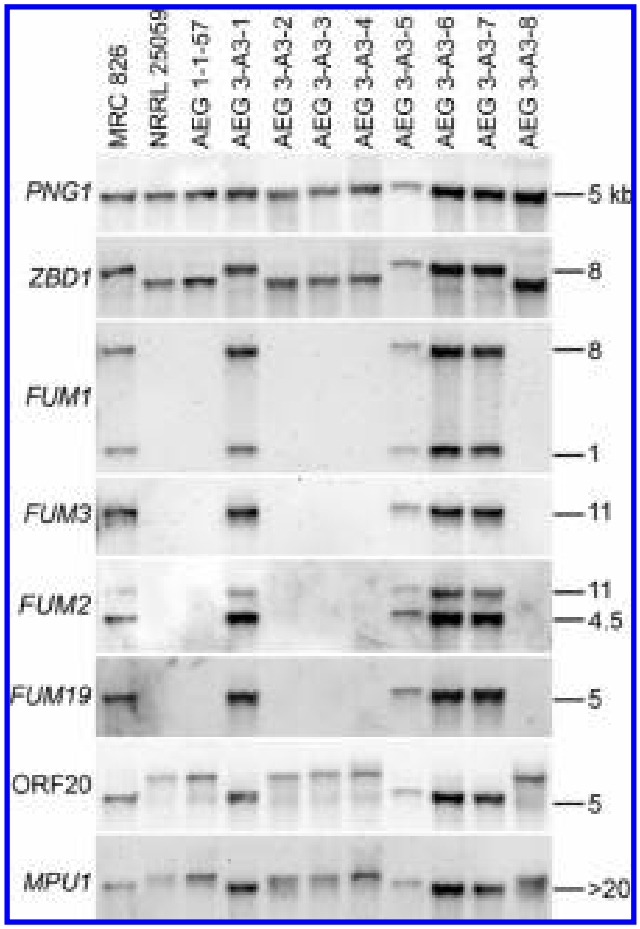

Fig. 1. Assessment of various *Fusarium verticillioides* strains by Southern hybridization for genes inside and outside the *FUM* cluster. *FUM1* is near the 5′ boundary of the cluster. *FUM19* is the final gene at the 3′ boundary of the cluster. *PNG1* and *ZBD1* are 5′ flanking genes upstream of the cluster, and ORF20 and *MPU1* are 3′ flanking genes downstream of the cluster (Proctor et al. 2003). *FUM3* and *FUM2* are within the cluster. *Eco*RI-digested genomic DNA (5 μg) from each strain was blotted to a nylon membrane and probed individually for the respective genes. Fragment sizes (kb) are indicated on the right margin.

Production of fumonisins by *F. verticillioides* is dependent on a biosynthetic gene (*FUM*) cluster of 16 contiguous and co-expressed genes encompassing approximately 45.7 kb on chromosome 1 (Brown et al. 2007; Proctor et al. 2003; Xu and Leslie 1996). *F. proliferatum*, another species commonly isolated from maize, also produces fumonisins (Logrieco et al. 2002; Ross et al. 1990) and has a *FUM* cluster that is perfectly syntenic with the *F. verticillioides FUM* cluster (Waalwijk et al. 2004). However, genomic regions flanking the two *FUM* gene clusters are highly dissimilar, suggesting two independent integration events at different chromosomal locations.

Although most strains of *F. verticillioides* produce the full complement of FB1, FB2, FB3, and FB4, strains with relatively rare fumonisin production phenotypes have been isolated from maize. These strains do not produce fumonisins or produce only FB2 and FB4 or FB3 and FB4 (Desjardins et al. 1996). Molecular genetic analysis indicated that the altered production phenotypes can result from mutations in genes within the *FUM* cluster. For example, the fumonisin-nonproduction phenotype of two maize strains of *F. verticillioides* resulted from mutations in *FUM1*, a polyketide synthase gene in the cluster (Proctor et al. 2006).

In contrast to strains from maize, fumonisin-nonproduction is the dominant phenotype among strains of *F. verticillioides* associated with banana fruit produced in Central and South American and the Canary Islands (Mirete et al. 2004; Moretti et al. 2004). These strains appear to be part of a distinct, relatively homogeneous population (Hirata et al. 2001; Mirete et al. 2004; Moretti et al. 2004). The molecular genetic basis for the lack of fumonisin production in these banana strains of *F. verticillioides* is not fully known, though some have suggested that the *FUM* cluster may be partially deleted (González-Jaén et al. 2004; Mirete et al. 2004). For example, Mirete and associates (2004) cited unpublished results from a Southern blot analysis in which probes prepared for two genes within the *FUM* cluster (*FUM1* and *FUM8*) did not hybridize to genomic DNA of some banana strains.

In the current study, we further characterized *F. verticillioides* strains from banana to determine the molecular genetic basis of their fumonisin-nonproducing phenotype. Specifically, we addressed the hypothesis that the *FUM* cluster is absent in banana strains. Furthermore, we tested the hypothesis that these fumonisin-nonproducing strains can be genetically complemented by transformation with the full suite of *FUM* genes. We further utilized these strains to address the hypothesis that fumonisin production is necessary for development of leaf lesions and other symptoms associated with *F. verticillioides*-induced maize seedling blight. The results support each of these hypotheses. The *FUM* cluster was absent from the banana strains we examined, and data were consistent with these strains possessing a unique deletion of the cluster, which we were able to reintroduce by transformation-mediated complementation. Results from maize seedling assays also suggested that fumonisin production by *F. verticillioides* was necessary for development of foliar disease symptoms.

RESULTS

FUM cluster analysis in banana strains.

F. verticillioides strains MRC 826 from maize and NRRL 25059 from banana were assessed by Southern hybridization for genes in the *FUM* cluster as well as genes flanking the cluster (Fig. 1). Genomic DNA from both strains hybridized with probes for the flanking genes, *PNG1* and *ZBD1* from the 5′ flank and ORF20 and *MPU1* from the 3′ flank. Although

genomic DNA of maize strain MRC 826 hybridized with probes for the *FUM* cluster genes (*FUM1*, *FUM3*, *FUM2*, and *FUM19*), no hybridization was detected between the same *FUM* gene probes and genomic DNA of banana strain NRRL 25059. Because the four probes represent regions at both ends and near the middle of the *FUM* cluster, lack of hybridization suggested that most, if not all, of the cluster was absent. Strain AEG 1-1-57, a progeny derived from a sexual cross between MRC 826 and NRRL 25059 (Table 1), exhibited a hybridization pattern identical to NRRL 25059 for all genes examined (Fig. 1). Strains AEG 3-A3-1 through AEG 3-A3-8 (Table 1), an octad of meiotically related progeny derived from a backcross of AEG 1-1-57 to MRC 826, exhibited hybridization patterns identical to either parental strain, with the patterns segregating 1:1 for either the presence of the *FUM* cluster (MRC 826 parental pattern) or its absence (NRRL 25059 parental pattern) (Fig. 1). Banana strains NRRL 13911, NRRL 13912, NRRL 13913, and NRRL 13914 also were examined by Southern analysis and exhibited the same hybridization pattern as NRRL 25059 (data not shown).

Nucleotide sequence analysis of genes *ZBD1* and ORF20 in banana strain NRRL 25059 revealed that their coding regions were only 5,395 bp apart (Fig. 2A). In contrast, the *ZBD1* and ORF20 coding regions are separated by 49 kb of intervening DNA in the reference sequence from maize strain FRC M-3125 (GenBank accession number AF155773). This intervening DNA encompasses the 16 genes of the *FUM* cluster, with *FUM21* and *FUM19* at the 5′ and 3′ ends of the cluster, respectively (Brown et al. 2007; Proctor et al. 2003). Further analysis of the nucleotide sequence of the *ZBD1*-ORF20 intergenic region in NRRL 25059 revealed the absence of almost the entire *FUM* cluster. The aberrant *FUM* locus in NRRL 25059 included 754 bp of the 5′ end of the *FUM21* coding region and 905 bp of the 3′ end of the *FUM19* coding region. The partial *FUM21* and *FUM19* sequences were contiguous with one another (Fig. 2A). Alignment to accession AF155773 indicated that 43.9 kb of the *FUM* cluster was absent in strain NRRL 25059. Strains FRC M-3125 and NRRL 25059 share 94.5 and 97.3% nucleotide sequence identity within the retained segments of *FUM21* and *FUM19*, respectively. The nucleotide sequence of the aberrant *FUM* locus of strain NRRL 25059 was deposited in GenBank (accession number EF653133).

FUM21 encodes a Zn(II)2Cys6 transcriptional regulator (Brown et al. 2007), whereas *FUM19* encodes an ABC transporter (Proctor et al. 2003). A search of expressed gene sequences in the *F. verticillioides* Gene Index using the 754 bp of NRRL 25059 *FUM21* identified TC30495 with the highest homology (*E* value = 3.0e-121). This TC is one of the alternative splice forms of *FUM21* from FRC M-3125 (Brown et al. 2007). Similarly, a search of the gene index using the 905 bp of *FUM19* from NRRL 25059 identified TC33164 (*E* value = 3.3e-147), which is the expressed sequence of *FUM19* from

Table 1. Characteristics of *Fusarium verticillioides* strains examined in this study

Strain[b]	Origin[c]	Mating type	*FUM* genotype[d]	Fumonisin production (μg/g)[a]			Incidence (%)[e]
				FB1	FB2	FB3	
MRC 826	Maize; South Africa	*MAT1-1*	*FUM*	364 ± 142	164 ± 70	24 ± 9.4	81 ± 13 bc
NRRL 25059	Banana; Honduras	*MAT1-2*	*Δfum*	nd	nd	nd	1.0 ± 2.0 g
AEG 1-1-57	MRC 826 × NRRL 25059	*MAT1-2*	*Δfum*	nd	nd	nd	1.0 ± 3.0 g
AEG 3-1-6	MRC 826 × AEG 1-1-57	*MAT1-2*	*Δfum*	nd	nd	nd	0.0 g
AEG 3-A3-1	MRC 826 × AEG 1-1-57	*MAT1-2*	*FUM*	28 ± 8.7	9.0 ± 2.8	4.0 ± 0.8	43 ± 28 f
AEG 3-A3-2	MRC 826 × AEG 1-1-57	*MAT1-2*	*Δfum*	nd	nd	nd	0.0 g
AEG 3-A3-3	MRC 826 × AEG 1-1-57	*MAT1-1*	*Δfum*	nd	nd	nd	0.0 g
AEG 3-A3-4	MRC 826 × AEG 1-1-57	*MAT1-1*	*Δfum*	nd	nd	nd	0.0 g
AEG 3-A3-5	MRC 826 × AEG 1-1-57	*MAT1-1*	*FUM*	163 ± 102	45 ± 29	34 ± 15	93 ± 3.0 ab
AEG 3-A3-6	MRC 826 × AEG 1-1-57	*MAT1-1*	*FUM*	193 ± 15	56 ± 5.3	38 ± 3.9	85 ± 16 abc
AEG 3-A3-7	MRC 826 × AEG 1-1-57	*MAT1-2*	*FUM*	144 ± 29	42 ± 9.1	16 ± 2.4	48 ± 11 f
AEG 3-A3-8	MRC 826 × AEG 1-1-57	*MAT1-2*	*Δfum*	nd	nd	nd	0.0 g
AEG 73-A4-2	MRC 826 × AEG 3-1-6	*MAT1-1*	*Δfum*	nd	nd	nd	0.0 g
RRC 1823	NRRL 25059::Cos4-5	*MAT1-2*	*Δfum/FUM7-19*	nd	nd	nd	0.0 g
RRC 1825	NRRL 25059::Cos6B/Cos4-5	*MAT1-2*	*Δfum/FUM21-19*	238 ± 25	52 ± 6.1	46 ± 3.8	97 ± 6.0 a
RRC 1827	NRRL 25059::Cos6B/Cos4-5	*MAT1-2*	*Δfum/FUM21-19*	700 ± 288	193 ± 89	99 ± 44	87 ± 12 abc
RRC 1829	NRRL 25059::Cos6B	*MAT1-2*	*Δfum/FUM21-7*	nd	nd	nd	0.0 g
RRC 1830	NRRL 25059::Cos6B/Cos4-5	*MAT1-2*	*Δfum/FUM21-19*	558 ± 165	129 ± 46	84 ± 23	67 ± 25 de
FRC M-3125	Maize; California, USA	*MAT1-1*	*FUM*	57 ± 25	8.1 ± 3.9	11 ± 6.8	55 ± 31 ef
FRC M-3703	Maize; Indiana, U.S.A.	*MAT1-2*	*FUM*	673 ± 79	212 ± 13	185 ± 8.6	82 ± 12 bc
FRC M-5500	Maize; Nepal	*MAT1-2*	*fum1-2*	nd	nd	nd	0.0 g
57-7-7	FRC M-3125 × FRC M-5500	*MAT1-1*	*fum1-2*	nd	nd	nd	0.0 g
GfA2364	FRC M-3125::Δ*FUM1*	*MAT1-1*	*ΔFUM1*	nd	nd	nd	0.0 g
GfA2617	57-7-7::*FUM1*	*MAT1-1*	*fum1-2/FUM1*	112 ± 17	25 ± 5.9	21 ± 2.4	77 ± 3.0 cd

[a] Strains were grown on cracked corn (5 g) for 7 days. Fumonisins were extracted using acidified acetonitrile:water and quantified as detailed in the methods section (average amount ± standard deviation); nd = not detected.

[b] Strain GfA2364 is a *FUM1* gene deletion mutant of strain FRC M-3125 (Proctor et al. 1999). Strain GfA2617 was generated by transforming strain 57-7-7 with a wild-type allele of *FUM1*, thus complementing the native nonfunctional *fum1-2* allele (Desjardins et al. 1992, 1996; Proctor et al. 2006). Additional information on strains is detailed elsewhere (Glenn et al. 2002, 2004; Leslie et al. 1992).

[c] AEG strains are meiotic progeny derived from the indicated sexual crosses. MRC 826 was the female parent.

[d] *FUM* = fumonisin-producing strains having an intact *FUM* cluster as determined in this study or previously demonstrated (Proctor et al. 2003, 2006); *Δfum* = fumonisin-nonproducing strains lacking the *FUM* gene cluster as shown in this study. Transformation of NRRL 25059 with Cos6B provided genes *FUM21*, *FUM1*, *FUM6*, and *FUM7* from the *FUM* gene cluster, and transformation with Cos4-5 provided genes *FUM7* through *FUM19*. Transformation with both cosmids conveyed the full complement of *FUM* genes; *fum1-2* = fumonisin-nonproducing strains having a mutant allele of *FUM1* (Desjardins et al. 1992, 1995, 1996; Proctor et al. 2006).

[e] Seedling disease incidence: mean percentage of diseased seedlings per replicate (± standard deviation). Ten seeds were sown per replicate (= pot) with three replicates per experiment. Each strain was assessed in multiple experiments (total of 6 to 15 replicates), except for RRC 1823 through RRC 1830, which were assessed only once to minimize handling. Thus, for most strains, the data are derived from 60 to 150 seedlings that were scored for development of foliar disease symptoms. Means followed by the same letters (a through g) were not significantly different ($P < 0.0001$) according to Duncan's multiple range test.

FRC M-3125. Comparison of these TC sequences to the aberrant *FUM21-FUM19* gene fusion of NRRL 25059 suggested that a stop codon 30 nucleotides into the *FUM19* sequence may prevent translation of a chimeric protein from the possible aberrant transcript. Lastly, if the NRRL 25059 *FUM21-FUM19* contiguous sequence arose through deletion of the *FUM* cluster, the putative breakpoint in wild-type *FUM21* occurred within exon 3, whereas the putative breakpoint in wild-type *FUM19* occurred at the 3′ end of the third intron.

Other banana strains of *F. verticillioides* were assessed by polymerase chain reaction (PCR) for the absence of the *FUM* cluster. Based on the NRRL 25059 nucleotide sequence, PCR primers FUMdelfor and FUMdelrev were designed to anneal to priming sites 99 bp upstream and 685 bp downstream of the *FUM21-FUM19* junction, respectively, and, as a result, were predicted to generate a 784-bp amplicon (Fig. 2A). PCR with this primer pair produced the anticipated 784-bp amplicon from all banana strains (Fig. 2B), whereas MRC 826 and other maize strains did not yield an amplicon, presumably due to the 44.7 kb of *FUM* cluster between the priming sites. The nucleotide sequences of amplicons from all banana strains were identical to that of strain NRRL 25059. Taken together, the Southern analysis and sequence data suggest that the population of *F. verticillioides* occurring on banana consists of strains in which 43.9 kb of the *FUM* cluster has been deleted.

Complementation of the *FUM* cluster deletion.

Maize strains MRC 826, FRC M-3125, and FRC M-3703 all produced significant levels of FB1, FB2, and FB3 when cultured on cracked maize kernels (Table 1). In contrast, the banana strains did not produce any detectable fumonisins in cracked maize cultures because they lacked the *FUM* cluster. Likewise, the ability of progeny derived from sexual crosses to produce fumonisins was dependent on whether they possessed the *FUM* cluster (Table 1). Molecular genetic complementation of fumonisin production was performed by transforming banana strain NRRL 25059 with two independent overlapping cosmids, Cos6B and Cos4-5, which together contained the full *FUM* cluster (Proctor et al. 2003). In all, 25 transformants were obtained and screened. Six of the transformants were not fully characterized due to inconclusive data. Transformants RRC 1823 and RRC 1829 are examples of the 13 transformants that carried only Cos4-5 or Cos6B, respectively, as confirmed by Southern hybridization (Fig. 3) and PCR amplification (data

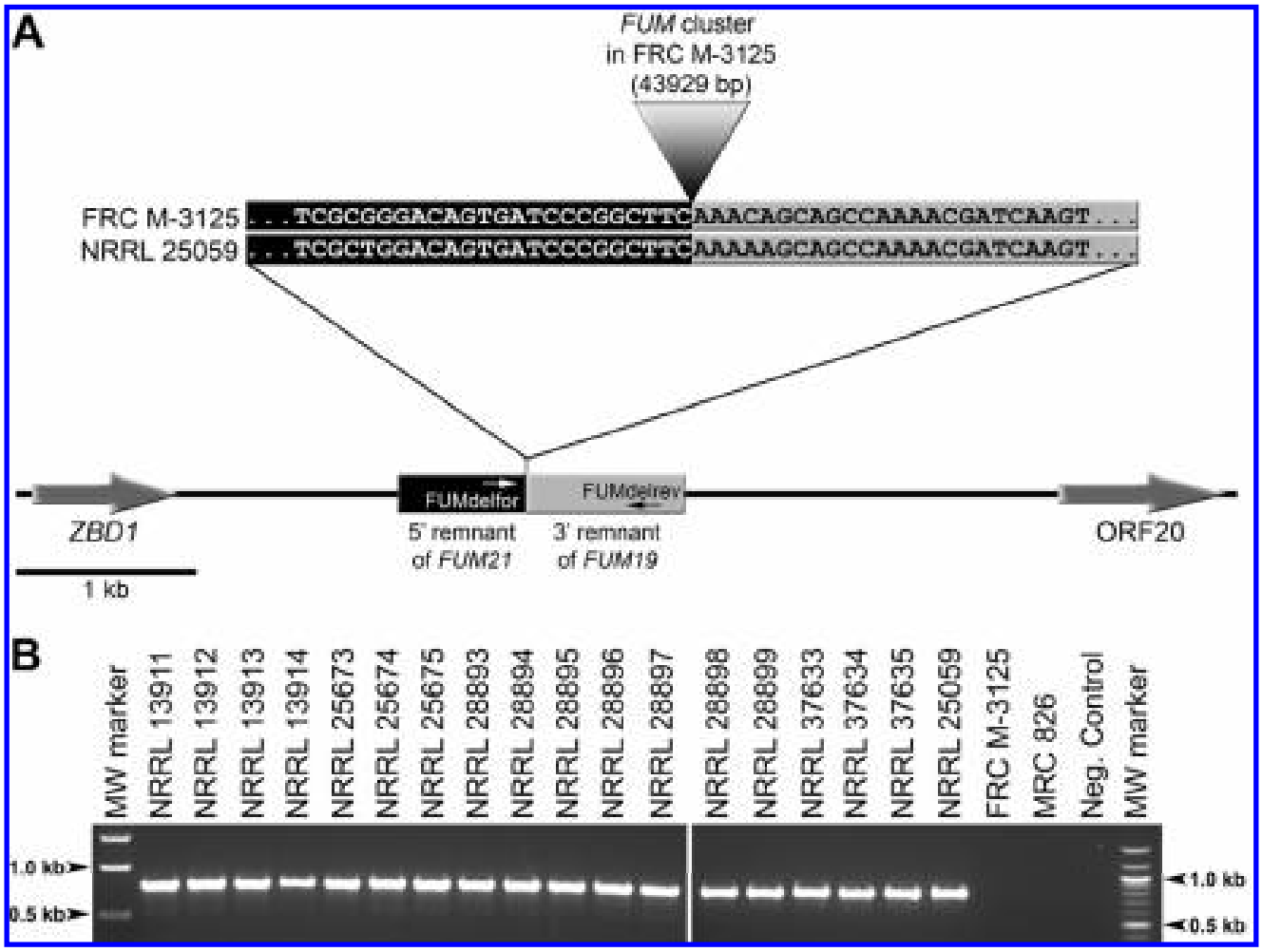

Fig. 2. A, Partial alignment of the *FUM* cluster nucleotide sequence from fumonisin-producing maize strain FRC M-3125 (GenBank accession no. AF155773) to the nucleotide sequence of the aberrant *FUM* locus from banana strain NRRL 25059 (accession no. EF653133). The sequence highlighted in black is within *FUM21*, and the sequence highlighted in gray is within *FUM19*. The triangle represents 43,929 bp of the *FUM* cluster present in strain FRC M-3125 that is absent in strain NRRL 25059. In the aberrant *FUM* locus of strain NRRL 25059, the 754-bp *FUM21* remnant is contiguous with the 905-bp *FUM19* remnant. The positions and orientations of primers FUMdelfor and FUMdelrev are indicated with small arrows. **B,** Polymerase chain reaction (PCR) assessment for absence of the *FUM* cluster in banana strains of *Fusarium verticillioides*. PCR with primers FUMdelfor and FUMdelrev generated a 784-bp amplicon from all banana strains, indicating that each strain lacked the *FUM* gene cluster. Strains FRC M-3125 and MRC 826 did not amplify a corresponding fragment due to the presence of the *FUM* cluster (44.7 kb) between the priming sites.

not shown). Neither RRC 1823 nor RRC 1829 produced fumonisins (Table 1). Cotransformants RRC 1825, RRC 1827, and RRC 1830 carried both cosmids (Fig. 3), which resulted in complementation of the *FUM* cluster deletion and production of FB1, FB2, and FB3 at levels similar to those produced by wild-type maize strains MRC 826, FRC M-3125, and FRC M-3703 (Table 1). In all, six cotransformants were identified and five were able to produce fumonisins. Thus, fumonisin production could be restored in strains lacking the *FUM* cluster by transformation-mediated introduction of cosmid clones that collectively carry the entire complement of *FUM* genes.

Fumonisin production and maize seedling disease.

Only strains of *F. verticillioides* that produced fumonisins caused foliar disease symptoms on seedlings of the maize hybrid 'Silver Queen' (Table 1). Strains that did not produce fumonisins did not cause foliar symptoms, and the seedlings from such treatments appeared equivalent to the uninoculated control seedlings. Disease symptoms included stunting of seedling growth, localized and coalescing necrotic lesions, mild bleaching of leaves, and atrophy of leaf tissue (Fig. 4). Necrotic lesions occurred mainly on the first and second leaves. Lesions were first evident 7 to 8 days after planting, with progressive necrosis thereafter (Fig. 4A through C). The bleaching and atrophy of marginal leaf tissue occurred on the third and subsequent leaves. The distal and proximal ends of the third leaf often were normal in appearance, with the bleaching and tissue atrophy localized to the middle of the leaf along its length (Fig. 4D). The atrophy on the third leaf often resulted in binding of the tips of subsequent leaves emerging from the whorl of the seedling, creating a tangle-top appearance (Fig. 4D, arrowhead).

The hybrid Silver Queen was used as the standard susceptible cultivar for our assays, but we also assessed foliar disease development on open-pollinated sweet corn lines 'Country Gentleman,' 'Golden Bantam,' and 'Stowell's Evergreen.' Inoculation of these three cultivars with *F. verticillioides* strain MRC 826 resulted in stunted seedlings with foliar disease symptoms as seen on Silver Queen. Each mean foliar disease incidence was 43, 78, and 92% of seedlings per replicate ($n = 6$ replicates), respectively. Two corn belt dents, inbred W23 and commercial hybrid P3223, also were screened for susceptibility and had a mean foliar disease incidence of 3.3 and 28% per replicate ($n = 6$ replicates), respectively; however, this included only mild bleaching of leaves without atrophy or lesion development and only mild stunting of the seedlings. Thus, maize genotype impacted both the incidence and severity of foliar disease development caused by pathogenic strain MRC 826.

An association between fumonisin production and pathogenicity was demonstrated by the transformation of banana strain NRRL 25059 with the overlapping cosmids as discussed above. NRRL 25059 was nonpathogenic on Silver Queen maize seedlings, as were transformants RRC 1823 and RRC 1829, which carried only one or the other of the two cosmids and did not produce fumonisins (Table 1; Fig. 5). However, cotransformants RRC 1825, RRC 1827, and RRC 1830, which carried both cosmid clones and produced fumonisins, caused significant disease incidence on maize seedlings (Table 1; Fig. 5). Thus, restoration of fumonisin production to the nonpathogenic strain NRRL 25059 by introduction of two cosmid clones carrying the entire complement of *FUM* genes also restored the ability of the strain to cause foliar disease symptoms on maize seedlings.

To further investigate the role of fumonisins in pathogenicity, we also examined previously characterized fumonisin-producing and fumonisin-nonproducing maize strains of *F. verticillioides* (Table 1). Wild-type maize strain FRC M-3125 produces FB1, FB2 and FB3, as noted above. Strain GfA2364 was derived from FRC M-3125 by transformation-mediated disruption of the *FUM1* gene and, as a result, does not produce fumonisins (Proctor et al. 1999). Strain 57-7-7 does not produce fumonisins because it carries a naturally occurring, nonfunctional allele of *FUM1* (Desjardins et al. 1996). Strain GfA2617 was derived from 57-7-7 by transformation with a wild-type copy of *FUM1* and as a result produces the wild-type complement of FB1, FB2, and FB3 (Proctor et al. 2006) (Table 1). Consistent with the data for transformants of banana strain NRRL 25059, fumonisin-producing strains FRC M-3125 and GfA2617 were pathogenic on maize seedlings, whereas the fumonisin-nonproducing strains GfA2364 and 57-7-7 were nonpathogenic (Table 1). Overall, the positive correlation between fumonisin production and pathogenicity on maize seedlings was consistent for *F. verticillioides* strains originating from both banana and maize.

To evaluate a correlation between presence and absence of the *FUM* cluster, fumonisin production, and pathogenicity, a random population of sexual progeny was assessed for both the production of fumonisins and the ability to cause maize seedling disease. Strain AEG 73-A4-2, which was derived from a second-generation backcross with MRC 826, lacked the *FUM* cluster, and did not produce fumonisins (Table 1), was sexually crossed with wild-type maize strain FRC M-3703. Of the 24 random ascospore progeny examined from this cross, 11 had the *FUM* cluster, produced fumonisins, and were pathogenic on maize seedlings (Fig. 6), whereas the remaining 13 progeny did not have the *FUM* cluster, did not produce fumonisins, and did not cause foliar disease symptoms on maize seedlings. The progeny ratio of 11:13 was consistent with the 1:1 segregation of a single locus ($0.7 > P > 0.6$). The 11 pathogenic progeny, along with their wild-type parental strain FRC M-3703, were assessed further for correlation of amount of

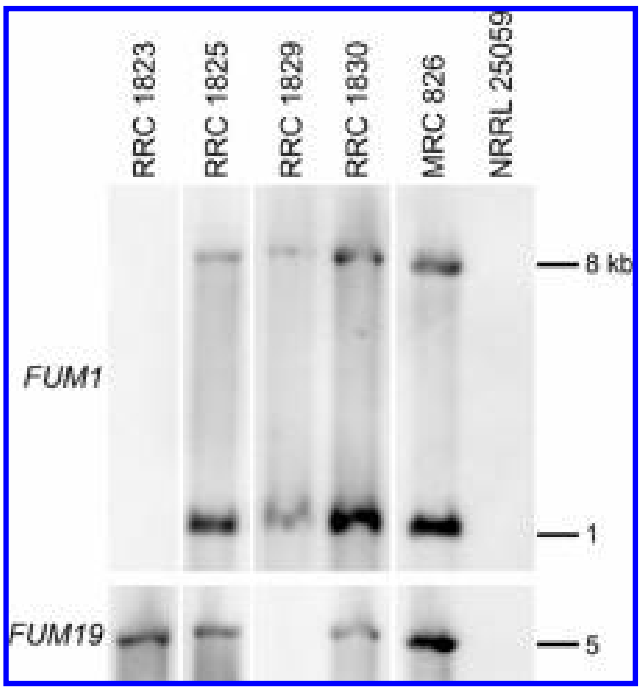

Fig. 3. Southern blot analysis of NRRL 25059 transformants with probes for the *FUM1* and *FUM19* genes. Transformant RRC 1823 received only one cosmid, Cos4-5, which carries *FUM7* to *FUM19*. Likewise, transformant RRC 1829 received only Cos6B, which carries *FUM21* to *FUM7*. Cotransformants RRC 1825 and RRC 1830 (and RRC 1827, not shown) received both cosmids. MRC 826 and NRRL 25059 are shown for comparison. *Eco*RI-digested genomic DNA (5 µg) from each strain was blotted to a nylon membrane and probed individually for the respective genes. Fragment sizes (kb) are indicated on the right margin.

FB1 produced on cracked maize kernels and the incidence of foliar disease symptoms on seedlings of Silver Queen. Fumonisin production was more controlled in this experiment than in the other routine assessments reported in Table 1. We added 1×10^6 conidia to the cracked corn, and replicate cultures of strain FRC M-3703 were collected on days 2 through 7 to determine the optimal sampling time point. Seven days after inoculation was sufficient for linear production of FB1 to substantial levels. A significant positive correlation between FB1 production and incidence of foliar disease symptoms for the 11 pathogenic progeny and parental strain FRC M-3703 is shown in Figure 6 ($R^2 = 0.66$; $P < 0.02$). Exclusion of parental strain FRC M-3703 from the analysis lowered the correlation significance ($R^2 = 0.59$; $P < 0.06$) but did not disrupt the trend.

DISCUSSION

Although *F. verticillioides* is best known for its worldwide occurrence on maize and the associated concern with fumonisin production, a population of this species that does not produce

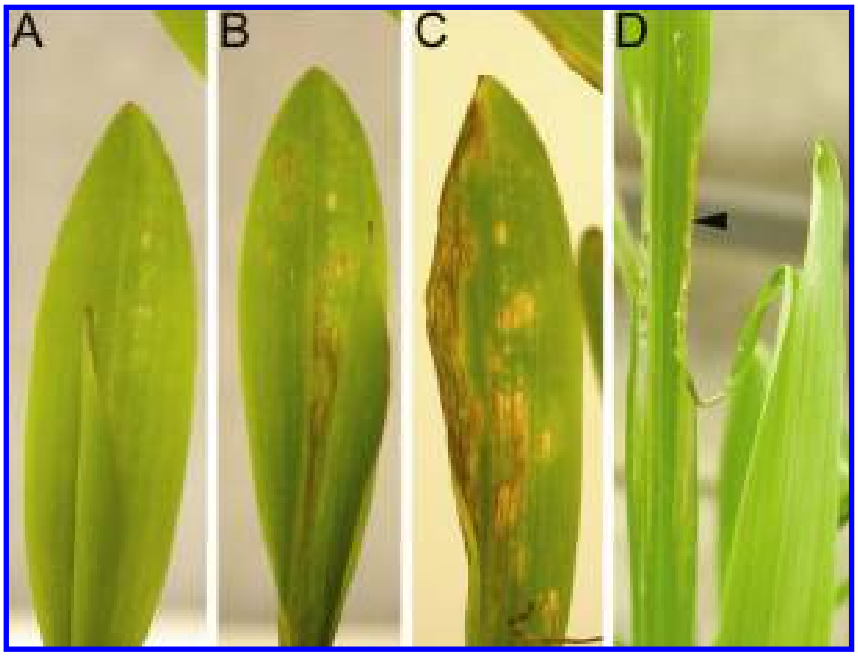

Fig. 4. Development of necrotic lesions, tissue atrophy, and mild bleaching on leaves of Silver Queen seedlings grown from seed inoculated with strain MRC 826 and planted in sterile potting soil. **A,** First leaf beginning to show necrotic lesions 7 days after planting (DAP). Second leaf is emerging from whorl. **B,** Same leaves with more extensive necrosis of the first leaf (8 DAP). **C,** First leaf with coalesced lesions (12 DAP). Second leaf was moved out of view to show full extent of necrosis on the first leaf. **D,** Third leaf (left) showing tissue atrophy and bleaching of the middle length of the leaf while the distal and proximal ends appear normal (14 DAP). The leaf tissue atrophy often caused binding of the tips of subsequent leaves emerging from the whorl of the seedling, creating a tangle-top appearance (arrowhead).

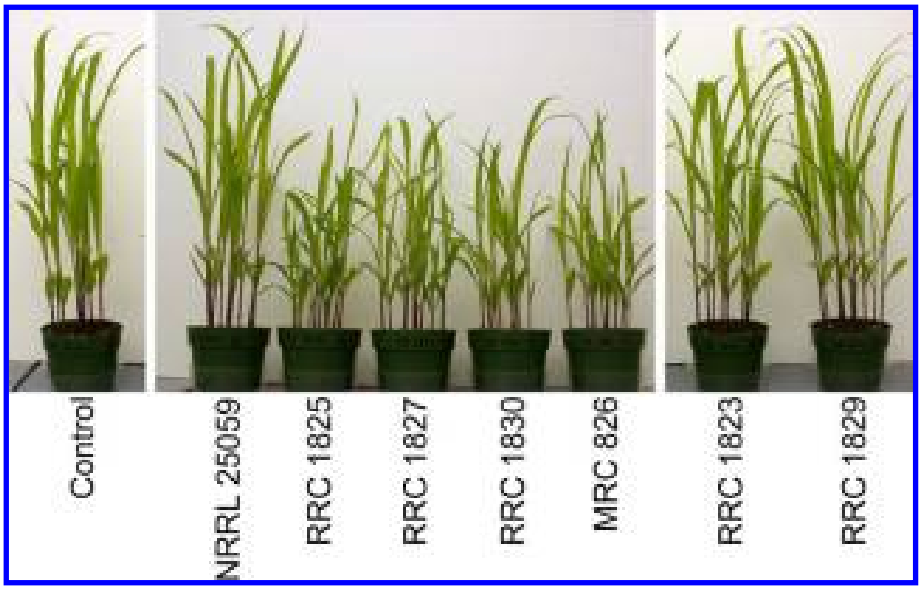

Fig. 5. Silver Queen seedlings (14 days after planting) grown from seed inoculated with the indicated strains. Seedlings inoculated with nonpathogenic strain NRRL 25059 were identical in appearance to the uninoculated control seedlings (no stunting or foliar disease symptoms). MRC 826 and the cotransformants RRC 1825, RRC 1827, and RRC 1830 were all pathogenic and caused stunting and foliar disease symptoms. Transformants RRC 1823 and RRC 1829 were nonpathogenic like NRRL 25059.

fumonisins is reported on banana. In this study, we investigated the genetic basis for the lack of fumonisin production by these banana strains of *F. verticillioides*. The data from Southern hybridizations, nucleotide sequencing, and PCR assessments suggest that the *FUM* cluster may have been deleted in *F. verticillioides* banana strains. Furthermore, transformation of a banana strain with the *FUM* cluster restored fumonisin production. These and additional strains also allowed us to demonstrate that fumonisin production is necessary for pathogenicity on maize seedlings, thus demonstrating that fumonisins can significantly impact maize–*F. verticillioides* interactions. Such results are consistent with our previous analyses of fumonisin bioavailability in soils and disruption of ceramide biosynthesis in maize seedlings (Williams et al. 2006, 2007).

Southern hybridizations indicated that fumonisin biosynthetic genes (*FUM1*, *FUM2*, *FUM3*, and *FUM19*) were absent in *F. verticillioides* banana strain NRRL 25059 and the fumonisin-nonproducing meiotic progeny derived from it. Similarly, Mirete and associates (2004) reported as unpublished data that *FUM1* and *FUM8* were not detected in a Southern hybridization analysis of *F. verticillioides* banana strains. *FUM1*, *FUM2*, *FUM3*, *FUM8*, and *FUM19* are distributed over the entire length of the *FUM* cluster (Proctor et al. 2003), suggesting that most, if not all, of the cluster was absent in banana strains. In contrast, Southern hybridization analysis of banana strains did detect flanking genes that are upstream (*PNG1* and *ZBD1*) and downstream (ORF20 and *MPU1*) of the *FUM* cluster in maize strains of *F. verticillioides*.

Genomic sequencing of the *ZBD1*-ORF20 intergenic region in strain NRRL 25059 and PCR amplification of the aberrant *FUM* locus from other banana strains of *F. verticillioides* provided evidence that the cluster not only was absent but may have been deleted from the strains. The banana strains retained 754 bp of the 5′ end of the *FUM21* coding region that was contiguous with 905 bp of the 3′ end of the *FUM19* coding region. In maize strains, these *FUM21* and *FUM19* sequences are separated by 43.9 kb that includes the rest of the *FUM* cluster genes. To our knowledge, this is the first report detailing at the nucleotide level a possible *FUM* gene cluster deletion in *F. verticillioides*. Analysis of the aberrant *FUM21-FUM19* gene sequence in NRRL 25059 suggested that it likely does not encode a chimera of a Zn(II)2Cys6 transcription factor and an ABC transporter.

Absence of the *FUM* cluster in banana strains of *F. verticillioides* is distinctly different from the ubiquitous presence of the cluster in maize strains. Although fumonisin-nonproducing natural variants have been isolated from maize (e.g., strains FRC M-5500 and FRC M-5550), they have defective *FUM1* alleles but otherwise have an intact *FUM* cluster (Desjardins et al. 1992, 1995, 1996; Proctor et al. 2006). Overall, the occurrence of maize strains of *F. verticillioides* that do not produce fumonisins is rare. In contrast, *Aspergillus flavus* strains that do not produce aflatoxins due to various deletions within its biosynthetic gene cluster are commonly isolated from maize and other agricultural hosts (Chang et al. 2005).

The *FUM* gene cluster itself may have a diverse evolutionary history. Waalwijk and associates (2004) found that the *FUM* gene clusters in *F. verticillioides* and *F. proliferatum* were perfectly syntenic, yet the region 5′ to *FUM21* was not orthologous in the two species. This finding suggests that the cluster is located at different positions within the genomes of these two species. Waalwijk and associates (2004) were unable to determine the genomic sequence of the 3′ flanking region for the *F. proliferatum FUM* cluster. They also noted that the higher-than-expected level of dissimilarity between corresponding genes within the two *FUM* clusters was supportive of independent acquisition events from distinct genetic sources. Given the discontinuous distribution of fumonisin production and the *FUM* genes within the *Gibberella fujikuroi* species complex (Proctor et al. 2004), such independent acquisitions may have occurred during the evolution of various species. Additionally, the apparent deletion of almost the entire *FUM* cluster in banana strains of *F. verticillioides* suggests that the discontinuous distribution of the cluster could be due in part to loss of the cluster during species differentiation.

We were able to introduce the *FUM* genes into banana strain NRRL 25059 through transformation-mediated complementation, thus making the normally fumonisin-nonproducing strain able to produce FB1, FB2, and FB3 to levels comparable with wild-type maize strains. Functional introduction into a filamentous fungus of an entire cluster of genes responsible for production of a class of secondary metabolites is a unique result of this study, especially because the full complement of genes was carried on two different cosmid clones. In contrast, Malonek and associates (2005) were able to restore gibberellin production in nonproducing strains of *F. proliferatum*, but this approach involved transformation with single genes cloned from the gibberellin-producing species *F. fujikuroi*.

It is worth noting that the strains of *F. verticillioides* isolated from banana fruit produced in Central and South America and the Canary Islands are considered members of this species based on a multigene phylogenetic analysis of *Fusarium* spp. within the *G. fujikuroi* complex, their morphology, and their sexual fertility with standard *F. verticillioides* mating strains (Glenn et al. 2002; Hirata et al. 2001; Moretti et al. 2004; O'Donnell et al. 1998). However, banana strains are not pathogenic on maize seedlings (Glenn et al. 2002; Hirata et al. 2001), do not produce fumonisins (Mirete et al. 2004), and are unable to tolerate or detoxify the maize antimicrobial compound 2-benzoxazolinone (BOA) (Glenn et al. 2001). Typical maize strains of *F. verticillioides* metabolically transform BOA into the nontoxic *N*-(2-hydroxyphenyl) malonamic acid (Glenn 2006; Glenn et al. 2001). Thus, banana strains appear to be a distinct population within *F. verticillioides* and could represent

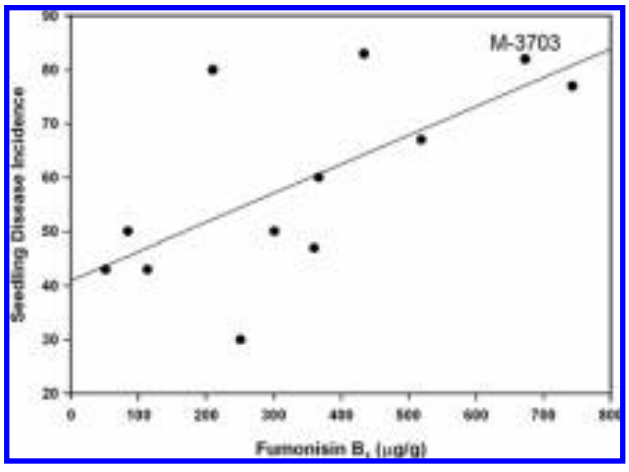

Fig. 6. Seedling disease incidence was positively correlated with the amount of FB1 produced by pathogenic strains of *Fusarium verticillioides*. From a cross between fumonisin-producing maize strain FRC M-3703 and fumonisin-nonproducing strain AEG 73-A4-2, 11 ascospore progeny produced fumonisins and were pathogenic, whereas the other 13 progeny did not produce fumonisins and were nonpathogenic. Plotted here is the maize seedling disease incidence for each of the 11 pathogenic progeny and parental strain FRC M-3703 versus the average amount of FB1 produced by the strains. A significant positive correlation was evident based on Pearson product moment correlation analysis ($R^2 = 0.66$; $P < 0.02$). Exclusion of parental strain FRC M-3703 from the analysis lowered the correlation significance ($R^2 = 0.59$; $P < 0.06$).

a closely related cryptic species (Hirata et al. 2001; Mirete et al. 2004; Moretti et al. 2004).

The near-ubiquitous production of fumonisins by *F. verticillioides* populations from maize suggests that the mycotoxins may confer a biological advantage, such as antibiosis against competing microbes or pathogenicity on its host plant. In this current study, we have demonstrated that *F. verticillioides* pathogenicity on Silver Queen seedlings, as defined by the ability to cause necrotic leaf lesions and other foliar disease symptoms, was dependent on fumonisin production. Other maize cultivars also were susceptible. The open-pollinated sweet corn lines were more susceptible to such disease development than the inbred and hybrid dent corn. A much broader assessment of seedling disease susceptibility among diverse maize genotypes is needed in order to determine whether foliar disease development is restricted to certain races or specific cultivars. For example, the great majority of North American sweet corn cultivars, including Country Gentleman, Stowell's Evergreen, Golden Bantam, and the hybrid Silver Queen, are phylogenetically related to Northern Flint races (Revilla and Tracy 1995). The susceptibility of Northern Flints and other maize races to fumonisin-related foliar disease development is currently being evaluated.

The most conclusive data supporting a link between fumonisin production and pathogenicity on maize seedlings came from analysis of a diverse collection of strains, including *FUM* gene complementation and deletion strains. Particularly interesting were the cosmid cotransformants (strains RRC 1825, RRC 1827, and RRC 1830) of *F. verticillioides* banana strain NRRL 25059 that produced significant levels of fumonisins and were pathogenic on maize seedlings. Additionally, disruption of the *FUM1* gene in wild-type maize strain FRC M-3125 (Proctor et al. 1999) resulted in a mutant (strain GfA2364) that did not produce fumonisins and also did not cause foliar disease symptoms on maize seedlings. Conversely, complementation of a native nonfunctional *FUM1* allele (*fum1-2*) in nonpathogenic strain 57-7-7 with the cloned wild-type *FUM1* allele from strain FRC M-3125 resulted in strain GfA2617 (Proctor et al. 2006) that was able to produce fumonisins and did cause foliar disease symptoms on maize seedlings.

We previously have shown a significant positive correlation between leaf lesion development on maize seedlings and the production of FB1 by *F. verticillioides* strains (Williams et al. 2006, 2007). Data also indicated a significant inverse correlation between amount of FB1 associated with seedling roots and the root weight and stalk height. In this current study, the analysis of 11 randomly segregating pathogenic progeny and their wild-type parental strain FRC M-3703 supported a significant positive correlation between the amount of FB1 produced by the strains and the level of disease incidence they were capable of causing on maize seedlings. Furthermore, we have shown that watering uninoculated maize seedlings with FB1 caused an inversely correlated dose-dependent reduction in root and stalk development and a positively correlated dose-dependent increase in leaf lesions (Williams et al. 2007). Roots from these plants also contained FB1 at concentrations positively correlated with the doses, indicating that FB1 could be absorbed by the roots (Williams et al. 2007). Such absorption resulted in inhibition of ceramide synthase, disruption of sphingolipid metabolism, and elevation of long-chain bases (sphinganine and phytosphingosine) and their 1-phosphates (sphinganine-1-phosphate and phytosphingosine-1-phosphate). Similarly, the susceptibility of tomato to FB1 and AAL-toxin was correlated with inhibition of ceramide synthase and disruption of sphingolipid metabolism (Abbas et al. 1994). Ceramide synthase inhibition by FB1, and the resulting perturbation of the sphingolipid biosynthetic pathway, is proposed to be an important factor for development of maize seedling blight disease because elevated cellular concentrations of long-chain bases or long-chain base 1-phosphates results in programmed cell death in plants (Lynch and Dunn 2004; Spassieva et al. 2002). Such fumonisin-induced programmed cell death may be the basis for development of foliar disease symptoms (e.g., leaf lesions) on maize seedlings. Furthermore, sphingolipids are essential components of plant cells contributing to seed viability, overall growth and development, and leaf morphology (Chen et al. 2006).

Together, our current and previous analyses help to refine the potential role of fumonisins in maize–*F. verticillioides* interactions (Abbas and Boyette 1992; Doehlert et al. 1994; Gilchrist et al. 1992; Lamprecht et al. 1994; van Asch et al. 1992; Williams et al. 2007). Desjardins and associates (1995) analyzed progeny from genetic crosses for a correlation between fumonisin production and virulence on maize seedlings, and they concluded that fumonisin production enhanced virulence but was not necessary or sufficient for maize seedling disease development. Differences in methodology between the current study and this previous report may be responsible for the different conclusions despite having used some of the same maize cultivars. For example, we planted into sterile potting soil seed that were inoculated with fungal spores, whereas Desjardins and associates (1995) planted uninoculated seed into coarse sand containing *Fusarium*-infested maize meal. Oren and associates (2003) found that, although seed inoculation did not suppress the growth of their seedlings, soil inoculation reduced plant growth, with greater reductions occurring at higher inoculum levels. We purposefully kept our inoculum concentration low (10^4 conidia/ml) so that virulence or pathogenicity factors were not potentially masked by necrotrophic fungal growth resulting from large inoculum levels. Additionally, potting and field soils are capable of binding and releasing biologically active FB1, whereas sand has minimal binding capacity for FB1, allowing the toxin to easily leach out (Williams et al. 2003, 2006). Thus, the impact of fumonisin production on disease development should be more pronounced in potting and field soils compared with sand.

Based on the results of the current study, which used a molecular genetic approach that analyzed strains derived by gene complementation and targeted gene disruption, we suggest that fumonisin production by *F. verticillioides* is necessary for development of foliar disease symptoms on seedlings of Silver Queen and other maize genotypes. The overall incidence and severity of seedling disease development are likely dependent on both the maize genotype and the amount of fumonisin produced by *F. verticillioides* strains. Furthermore, this study supports the hypothesis that FB1 may have an ancestral function as a phytotoxin (Desjardins et al. 2005). In addition to a thorough assessment of maize genotypes for susceptibility to fumonisin-related seedling disease, more research is needed on host defense mechanisms associated with responses to fumonisin, including determination of whether FB1 is translocated within maize or whether some other molecule serves as a mobile signal for cellular responses in leaves.

MATERIALS AND METHODS

Fungal strains and genetic crosses.

F. verticillioides strains used in this study that originally were isolated from bananas included NRRL 25059 (Table 1), NRRL 13911 to NRRL 13914 (from store-bought fruit in Peoria, IL, U.S.A.), NRRL 25673 to NRRL 25675 (from fruit produced in Guatemala), NRRL 28893 to NRRL 28899 (other accessions = MAFF 237853 to MAFF 237859, respectively, from fruit produced in Mexico and exported to Japan), and NRRL 37633 to NRRL 37635 (from fruit produced in Guatemala). Information on other *F. verticillioides* strains used in this study is detailed in Table 1. An octad of progeny (strains

AEG 3-A3-1 through AEG 3-A3-8) was collected from a single ascus from a sexual cross of strains MRC 826 and AEG 1-1-57. The twin pairs resulting from post-meiotic mitosis were AEG 3-A3-1/AEG 3-A3-7, AEG 3-A3-2/AEG 3-A3-8, AEG 3-A3-3/AEG 3-A3-4, and AEG 3-A3-5/AEG 3-A3-6.

For long-term storage of strains, conidia or hyphae were frozen at –80°C in 15% glycerol. For routine culturing, strains were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit) or in potato dextrose broth (PDB; Difco Laboratories) and incubated at 27°C in the dark, with the addition of shaking (200 rpm) for PDB cultures. Standard procedures were used for performing genetic crosses and collecting progeny (Glenn et al. 2002; Leslie and Summerell 2006).

***FUM* gene cluster analysis and transformation.**

Genomic DNA of *F. verticillioides* strains was extracted from 4- to 7-day-old PDB liquid cultures using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's protocol. Strains were assessed by Southern hybridization for the presence of *FUM* cluster genes (*FUM1*, *FUM3*, *FUM2*, and *FUM19*) and genes flanking the cluster (*PNG1*, *ZBD1*, ORF20, and *MPU1*) (Proctor et al. 2003, 2006). Southern hybridizations were performed by blotting *Eco*RI-digested genomic DNA (5 µg) from a 1.0% Tris-acetate-EDTA agarose gel onto a Hybond-N+ nylon membrane (Amersham Biosciences, Buckinghamshire, England) according to standard procedures. Gene-specific probes were generated using a digoxigenin (DIG) PCR labeling kit (Roche, Indianapolis, IN, U.S.A.). Primers for generation of the probes were as follows: *PNG1* (PNG1for = 5′-ATGCTCTGCTCAACATTGTGGTGCTT-3′; PNG1rev = 5′-AATCGCTCGTCCTTGTTCAT-3′), *ZBD1* (ZBD1for = 5′-GCAGTCTACGGCATGGAGTT-3′; ZBD1rev = 5′-GGTGGAGAATTGGGTGCTTA-3′), *FUM1* (FUM1for = 5′-TGAGATAAACATGATCTTCACTGGA-3′; FUM1rev = 5′-CGTGTATACTGCTGATTGACATAGC-3′), *FUM3* (FUM3for = 5′-ATCTAACCCAAGATGTAAAGCCAAT-3′; FUM3rev = 5′-CTCATCAGTGGAAAAGTCATTCAC-3′), *FUM2* (FUM2for = 5′-AAGAACCTCTGCTGTCCAAG-3′; FUM2rev = 5′-CATGAAGATGTCGGCATTAG-3′), *FUM19* (FUM19for = 5′-CTGCAGCATCAAGGAGAACA-3′; FUM19rev = 5′-GCTTTGCAAATGGCTTTGAT-3′), ORF20 (ORF20for = 5′-TAGTGAACTTTCGGCTGTCA-3′; ORF20rev = 5′-AGACAGCTTGGATCTTGACC-3′), and *MPU1* (MPU1for = 5′-TTGCTGGAAACGCTCAGAA-3′; MPU1rev = 5′-ATCGGCGAGACCTGCTACAAAT-3′). Reactions consisted of 1.0 unit of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1× PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, and 10 ng of genomic DNA prepared from *F. verticillioides* strain MRC 826. Thermal cycling consisted of initial denaturation at 95ºC for 5 min; followed by 40 cycles of 95ºC for 10 s, 60ºC for 10 s, and 72ºC for 30 to 45 s, depending on the amplicon size; and a final incubation at 72°C for 5 min. To confirm proper amplification, unlabeled amplicons were cleaned (QIAquick PCR purification kit, Qiagen Sciences) and sequenced either at the United States Department of Agriculture–Agricultural Research Service South Atlantic Area Sequencing Facility (Athens, GA, U.S.A.) or the Eastern Regional Research Center Nucleic Acid Facility (Wyndmoor, PA, U.S.A.). Each probe (120 µl) was diluted in 15 ml of DIG Easy Hyb solution (Roche) and heated to 100°C for 5 min. Hybridization and chemiluminescent detection of membranes were performed using Roche solutions and recommended procedures. An Alpha Innotech FluorChem 8000 digital imaging system (San Leandro, CA, U.S.A.) was used for visualization. Exposures of 50 min typically were required.

The genomic sequence of strain NRRL 25059 between *ZBD1* and ORF20 was determined using the GenomeWalker Universal Kit (Clontech Laboratories, Inc., Mountain View, CA, U.S.A.). Following the manufacturer's instructions, four libraries were constructed by digesting genomic DNA with *Eco*RV, *Dra*I, *Pvu*II, and *Stu*I followed by ligation of the GenomeWalker adaptor. Primary PCR of the four libraries was performed using the kit's AP1 adaptor primer with the respective gene-specific primers ZBD1-1 (5′-TCAGTGCATCTGGCAACACGGCCATCCA-3′) and ORF20-1 (5′-ATGCGCATGGCTAAAGTCACATCTCGGCCA-3′). Secondary PCR employed adaptor primer AP2 with ZBD1-2 (5′-AACAGTCTCGACGCCAAAGATGGGCTTGGT-3′) and ORF20-2 (5′-TGCAATCCATATGGCAGCCGATTGCAGGGT-3′). All final amplicons were cloned using TOPO TA Cloning Kit (Invitrogen). The secondary PCR primers were used to sequence the isolated clones. When necessary, the sequence data obtained with these primers was used to design additional primers to sequence along the entire length of the cloned amplicons. Sequence data were assembled and aligned to the fumonisin biosynthetic gene cluster (Proctor et al. 2003) (GenBank accession number AF155773) using Sequencher v.4.7 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). The assembled sequence from NRRL 25059 also was evaluated in comparison to tentative consensus sequences obtained from the *F. verticillioides* Gene Index at the Dana-Farber Cancer Institute.

The primer pair FUMdelfor (5′-GCCATTGTGCAAACCCTTTC-3′) and FUMdelrev (5′-CGTTGGATAACCCTGTGCAT-3′) was used to amplify across the location of the *FUM* cluster deletion in strain NRRL 25059. The PCR conditions noted above were used. All banana strains were screened for the anticipated 784-bp amplicon. The amplicons were sequenced for comparison to NRRL 25059.

Generation of protoplasts and polyethylene glycol-mediated transformations were performed as previously described (Proctor et al. 1999), with the following modifications. Cell wall digestion of germinated conidia was performed in a solution of 1.2 M $MgSO_4$ and 50 mM Na_3-citrate dihydrate (pH 5.8) containing Glucanex lysing enzyme (Sigma-Aldrich, St. Louis) at 13 mg/ml and β-glucuronidase (Sigma-Aldrich) at 2 mg/ml. Protoplasts were harvested by carefully overlaying an equal volume of ST solution (0.6 M sorbitol and 100 mM Tris-HCl, pH 7.4), followed by centrifugation (2,000 × *g*) in a swing bucket rotor for 20 min, and finally pipetting the protoplasts from the interface between the two solutions. Cotransformation of protoplasts from *F. verticillioides* strain NRRL 25059 was performed using two cosmids, Cos6B and Cos4-5, which together contain the full complement of fumonisin biosynthetic genes (Proctor et al. 2003). Because both cosmids carry the *hph* gene encoding hygromycin B phosphotransferase, hygromycin-resistant transformants were screened by Southern hybridization as described above using *FUM1* and *FUM19* probes to identify transformants that received either one of the cosmids or both cosmids. Final concentration of hygromycin B (Roche) in the transformation selection medium was 150 µg/ml. Hygromycin B was added to PDA or PDB (100 µg/ml) to maintain selection of transformants during subsequent propagation.

Fumonisin production.

Autoclaved cracked maize kernels (5 g hydrated with 50% water (vol/wt) in a 20-ml scintillation vial) were inoculated with a fungal suspension. For routine assessment of fumonisin production, approximately 2.5×10^7 conidia or hyphal clumps, depending upon the strain, were added to each vial. For a more detailed assessment of production kinetics, 1×10^6 conidia were added to each vial. Three vials of corn were inoculated for each strain. After 7 days of incubation at 27ºC in the dark, 10 ml of acetonitrile/water (1:1) containing 5% formic acid were added to each vial, which was shaken vigorously to dis-

rupt the fungal colony and placed on a rocker shaker for 3 h. Extracts were diluted 1,000-fold in acetonitrile/water (3:7) containing 1% formic acid. The diluted samples were analyzed by liquid chromatography-mass spectrometry as previously described (Williams et al. 2006).

Maize seedling disease assays.

Untreated seed of Silver Queen, Stowell's Evergreen, Country Gentleman, and Golden Bantam were obtained from W. Atlee Burpee & Co. (Warminster, PA, U.S.A.). Untreated seed of hybrid P3223 and inbred W23 were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA, U.S.A.) and the North Central Regional Plant Introduction Station (Ames, IA, U.S.A.), respectively. Seed were surface-disinfested for 10 min in 100% commercial bleach (5.25% sodium hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water (Glenn et al. 2002). The seed then were subjected to a heat shock treatment (60°C for 5 min) for internal sterilization (Bacon et al. 1994). Inoculations were performed by placing 40 sterilized seed in a 100-mm petri plate and flooding them with 10 ml of a conidial suspension (10^4 conidia/ml) as previously described (Glenn 2006). Control seed were treated with sterile water. Seed then were incubated overnight in the dark at 27ºC. Three replicates of 10 seed each were planted in sterile 10-cm plastic azalea pots containing twice-autoclaved growing mix (45% sphagnum peat; Conrad Fafard Inc., Agawam, MA, U.S.A.). Some uninoculated control seed were reserved for germination in sterile water to assess sterilization effectiveness. The replicates were arranged in a complete randomized block design in which fungal strain represented the block. Pots were watered from below 2, 4, and 6 days after planting and then from above as needed during the remainder of the assay. Each pot was placed in a plastic saucer to prevent cross-contamination during watering. Assays were performed in an environmental growth room cycling between 30°C (14-h day) and 20°C (10-h night). Disease symptoms were assessed 14 days after planting. The number of surviving plants, number of plants with leaf lesions, and number of plants with leaf atrophy or bleaching were recorded for each treatment replicate. Each fungal strain was assessed in multiple experiments for a total of 6 to 15 replicates, except for RRC 1823 through RRC 1830, which were assessed only once to minimize handling ($n = 3$ replicates). Thus, for most strains, the data are derived from 60 to 150 seedlings that were scored for development of foliar disease symptoms. Data were reported as mean disease incidence, which is the mean percentage of seedlings per replicate with leaf lesions or abnormalities.

Statistical analyses.

Statistical analyses of seedling disease data were performed using the GLM procedure of SAS System for Windows (version 8.2; SAS Institute, Cary, NC, U.S.A.). Treatments were evaluated for significance by analysis of variance. Data are presented as mean ± standard deviation, and differences between means were considered significant if probability (P) ≤ 0.05. Duncan's multiple range test was used for determination of means separation. The Pearson product moment correlation was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, U.S.A.) and used to measure the strength of the association between the amount of FB1 produced by strains grown on cracked maize kernels and the incidence of maize seedling disease caused by those strains.

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